Developing in biomarker blood test at 28 weeks gestation to identify pregnancies at high risk of fetal growth restriction and stillbirth

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SYNPOSIS OF PROJECT

Fetal growth restriction (FGR) is associated with a very significantly increased risk of stillbirth. Therefore, it is conceivable that a non-invasive test that helps clinicians better identify pregnancies with unsuspected FGR could prevent stillbirths. It would allow clinicians to identify undiagnosed cases and offer timely delivery before stillbirth occurs.

In this grant, we propose developing a blood test that can be done at 26-28 weeks gestation, added to the existing test for gestational diabetes, to identify pregnancies at high risk of developing FGR.

Specifically, we proposed measuring free mRNA in maternal circulation, of placental origin, that may be expressed at different levels in the blood of women at 26-28 weeks who are destined to have an FGR fetus at birth. The vision is to develop a blood test that can identify pregnancies are destined to deliver low birthweight fetuses. Such pregnancies are at higher risk of stillbirth while the fetus remains in utero.

In work previous to this grant, we had already prospectively collected 600 samples of blood at 26-28 weeks. Using these samples already collected, we proposed in this application to now validate 11 promising mRNA biomarkers in these blood samples. We have recently published papers suggesting these 11 candidate genes may have promise as blood biomarkers identifying FGR in utero (1, 2).

Here, we are pleased to provide our final report.

OUR STATED HYPOTHESIS AND AIMS

HYPOTHESIS:

Measuring genes coding IGFs and/or placental specific genes in the maternal circulation at 28 weeks can generate a test that predicts pregnancies that will be complicated by fetal growth restriction (FGR).

AIMS:

1. To undertake a prospective cohort study, examining whether circulating mRNA that code IGF2, GH2 , IGFBP2 in the maternal circulation at 28 weeks gestation can predict FGR at term.
2. To undertake a prospective cohort study, examining whether circulating mRNA that code 8 placental specific genes in the maternal circulation at 28 weeks gestation can predict FGR at term.
3. To generate the best test that predicts FGR at term, using a combination of the best performing genes from aims 1 and 2.

The project has been completed as proposed.

FINAL PROGRESS REPORT – WHAT WE HAVE DONE

1. Clinical characterization

We had already prospectively collected 600 samples over 2-3 years (collected done prior to starting this current project).

Over the first 6 months of 2013, we characterized the clinical information on this sizeable cohort. This was a significant undertaking, where we recorded a large number of datapoints for every participant. For the FGR cohort, we obtained their medical records and recorded >100 datapoints (including neonatal outcomes) onto an electronic spreadsheet.

As noted in our previous progress report, we successfully collected clinical details on the 600 participants.

Among these 600 samples we identified 40 samples where bloods were taken at 28 weeks gestation where the fetuses ended up as FGR at birth (birthweight <5th centile at delivery). We identified 80 control samples, bloods taken at 28 weeks gestation where the fetus was of normal birthweight.

Hence, we identified a case control set of 120 samples (40 cases of FGR, and 120 controls) for further study.

2. Extraction of RNA from the blood samples

We performed mass extraction of RNA from all 600 samples. This was performed, in batch, using a robotic RNA extraction machine, The QIACube (Invitrogen). All RNA samples have been aliquoted and stored in -80 degrees. The purity and quantity of each tube has been confirmed.

3. PCR to screen for mRNAs in the maternal circulation that are differentially expressed at 28 weeks gestation in women destined to deliver FGR at term.

We measured the mRNA coding 11 candidate biomarkers in all blood samples (obtained at 26-28 weeks gestation) in the entire cohort. The intention was to validate their potential in predicting FGR in later pregnancy.

As discussed in the original project proposal, we searched for biomarkers that can predict term FGR (ie mRNAs of genes that were differentially regulated in the n=40 term FGR cases compared to controls):

We measured the following, using maternal bloods obtained from the n=120 case control set:

- 3 genes that code growth factors (IGF2, GH2, IGFBP2)
- 8 genes that code placental specific genes (ie genes that are very highly upregulated in placenta: CRH, PLAC3, TAC3, CSH1, Syncytin, PSG 1, PLAC 4 and KISS 1).
- 36 other placental specific genes using a PCR microarray (see our 6th month report for further details).

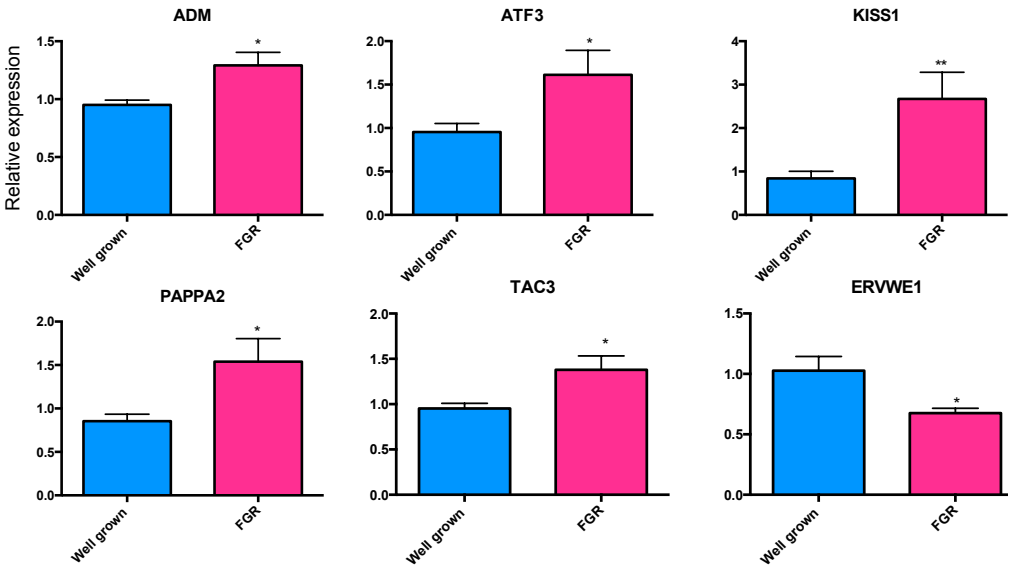
For these analyses', we identified the mRNA of the following six genes were differentially regulated in the maternal circulation among women destined to develop term FGR in this prospective cohort of n=120:

- Adrenomedullin (Adm): A hypoxic-induced gene that is very highly expressed in placenta relative to other tissues.

- Activating Transcription Factor 3 (ATF3): A gene involved in the innate immune system, but also highly expressed in placenta.
- Kisspeptin (KISS1): A gene known to be involved in the hypothalamic-pituitary axis, but also highly expressed in placenta (its biological role in placenta is unknown).
- Pappalysin 2 (PAPPA2): A gene that is highly expressed in placenta and probably regulates growth factor proteins.
- Tachykinin 3 (TAC3): A neurotransmitter that is highly expressed in placenta (biological function in placenta is unknown).
- Syncytin (or ERVWE1): Highly expressed in placenta, involved in placental cell biology where cytotrophoblasts differentiates to become the syncytiotrophoblast (the syncytiotrophoblast is the outer layer of the placenta that abuts the maternal circulation and produces a lot of the placental hormones).

Figure 1: mRNA Expression of six genes that were significantly differentially regulated in the maternal circulation at 28 weeks among women destined to deliver FGR at term.

FGR: n=40, defined as birthweight <5th centile at delivery (at term).
Well grown: n=80, defined as birthweight >20th centile (at term). *=p<0.05; ** p<0.01



4. Bioinformatics to identify whether a combination of these mRNAs could be used to develop a predict biomarker test.

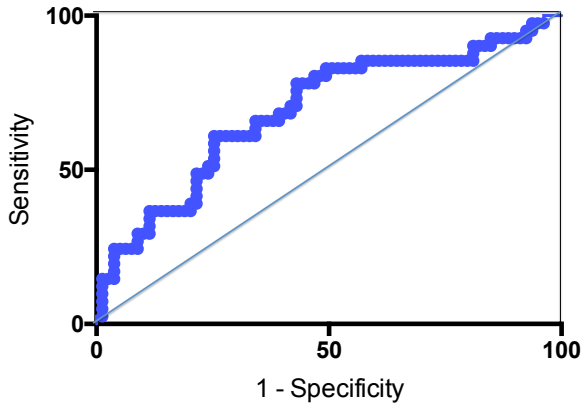
We examined the possibility of developing a multi-modal biomarker test using a combination of these six genes. We found the best test that can predict term FGR uses a combination of mRNA coding ATF3, ADM, KISS1 and TAC3 measured in the maternal blood at 28 weeks gestation.

A test that combines the results of the mRNA levels of these four genes yields a test that has a sensitivity of 67% and a specificity of 75%. Table 1 details the diagnostic performance of such a test.

Table 1: Diagnostic performance of a test to predict term FGR that combines the mRNA results of ATF3, ADM, KISS1 and TAC3 (measured in the maternal circulation at 28 weeks).

Diagnostic performance metrics (95%CI)	
Sensitivity	67% (50-81)
Specificity	75% (64-84)
Positive likelihood ratio	2.7 (1.8-4.2)
Negative likelihood ratio	0.4 (0.3-0.7)
Positive predictive value	57% (42-71)
Negative predictive value	82% (71-90)

Figure 2: ROC curve of a test to predict term FGR that combines the mRNA results of ATF3, ADM, KISS1 and TAC3 (measured in the maternal circulation at 28 weeks).



SUMMARY / CONCLUSION

Fetal growth restriction (FGR) can reflect a clinical situation where the placenta is working poorly, leading to a fetus that is suboptimally grown. *Importantly, FGR is strongly associated with stillbirth risk.* It is therefore an important clinical surrogate marker of stillbirth.

In this study, we set out to measure mRNA in the mum's blood at 28 weeks that could identify those at higher risk of developing FGR later in the pregnancy. For such pregnancies, the clinicians could offer timely delivery and thereby decrease the burden of stillbirth.

- In this work funded by The Stillbirth Foundation, *we have identified mRNA coding six genes are differentially expressed in the blood of mothers as early as 28 weeks, who are destined to develop FGR at term*.

- *This test can identify 67% of all cases of term FGR* (sensitivity of 67%).

- The accurately of this test probably just falls short of being sufficiently accurate to be adopted clinically. However, it forms the basis of a predict test where further studies could find other molecules to enhance its accuracy.

  - We are undertaking exactly such further studies. Using these same samples, we plan to undertake genomewide microarray studies to look more broadly, to find new mRNAs that may be differentially regulated in association with FGR. We are also undertaking other microarray studies to look at a different RNA species called microRNAs.

- We believe this work provides an important fundamental biological insight into FGR that is clinically noticed only at term. *Our data suggests that the biological process of FGR is already happening by 28 weeks gestation.* This opens the door to the rationale of developing therapies that could be applied during the last trimester of pregnancy since the pathology causing term FGR is already unfolding during this period. We think this is a novel insight into the biological development of FGR.

- *We also believe this work identifies six important molecules that may be involved in the biology of FGR.* We are now actively undertaking further laboratory studies to further examine what biological roles these molecules may have at the level of the placenta. It is possible that such studies could enhance our biological understanding how FGR (and hence stillbirth) develops.

We thank The Stillbirth Foundation for generously funding this work which we hope yields important insight into the field of Stillbirth Investigation.

REFERENCES:

1. **Whitehead CL, Walker SP, Ye L, Mendis S, Kaitu'u-Lino TJ, Lappas M, Tong S** 2013 Placental Specific mRNA in the Maternal Circulation Are Globally Dysregulated in Pregnancies Complicated by Fetal Growth Restriction. J Clin Endocrinol Metab
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